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Morphological and molecular characterisation of *Paralongidorus plesioepimikis* n. sp. (Nematoda: Longidoridae) from southern Spain

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Summary - *Paralongidorus plesioepimikis* n. sp. is described and illustrated by light microscopy, scanning electron microscopy and molecular studies from specimens collected in a sandy soil in the rhizosphere of stone pine (*Pinus pinea*) in Bonares (Huelva Province), southern Spain. The new species is characterised by a very long body (9.71-14.11 mm), a rounded lip region, with a clear constriction followed by a depression posterior to the amphidial aperture, a very long and flexible odontostyle (202-227 μm), guiding ring located at 36.5-44.0 μm from anterior end, dorsal pharyngeal gland nucleus in anterior part of bulb, one subventral pair of nuclei near middle of bulb, vulva at 33-38%, and a dorsally convex tail with rounded terminus (32-39 μm long). SEM observations showed a very large amphidial fovea with conspicuous aperture *ca* three-fourths as wide as lip region and cephalic papillae appearing as small apertures, each located just anterior to a distinct cephalic lobe. The sequences of the D2-D3 expansion segments of 28S rRNA, partial 18S rRNA and ITS rRNA gene for *P. plesioepimikis* n. sp. were obtained. Phylogenetic analyses of *P. plesioepimikis* n. sp. rRNA gene sequences and of *Paralongidorus* spp. and *Longidorus* spp. sequences published in GenBank were done using Maximum Likelihood and Bayesian inference. *Paralongidorus* species (including *P. plesioepimikis* n. sp.) clustered together, except for *P. bikanerensis* which clustered within *Longidorus* spp. and was clearly separated from all other *Paralongidorus* spp. in trees generated from the D2-D3 expansion segments of 28S and partial 18S data set. ML analysis using SH-test for the validity of *Paralongidorus* was performed and showed the validity of the genus using the D2-D3 expansion segment of 28S and partial 18S.

Keywords – description, molecular, morphometrics, morphology, needle nematode, new species, phylogeny, taxonomy.

Paralongidorus tends to be greatly conserved in gross morphology which makes species identification very challenging. Currently, species discrimination in *Paralongidorus* is mainly based on morphometrics and also morphological features referring to polytomous key (Escuer & Arias, 1997). Five species: *Paralongidorus bikanerensis* (Lal & Mathur, 1987) Siddiqi, Baujard & Mounport, 1993; *P. iranicus* Pedram, Pourjam, Namjou, Atighi, Cantalapiedra-Navarrete, Liébanas, Palomares-Rius & Castillo, 2012; *P. litoralis* Palomares-Rius, Subbotin, Landa, Vovlas & Castillo, 2008; *P. maximus* (Bütschli, 1874) Siddiqi, 1964, and *P. paramaximus* Heyns, 1965, have been molecularly characterised and so provide additional data for species identification.

The polytomous key for *Paralongidorus* spp. (Escuer & Arias, 1997) is an effective means for identifying species as it allows a range of characters to be used simultaneously. This key makes it more effective for identifying closely related species with overlapping features (Escuer & Arias, 1997). *Paralongidorus* comprises about 90 nominal species of migratory ectoparasites that spend their entire life cycle outside the host plant roots, and are of special scientific and economic interest because they directly damage the roots of the host plant and some species are vectors of economically important pathogenic plant viruses (Decraemer & Robbins, 2007).

Paralongidorus is well established and widely accepted by nematologists, although its definition is controversial as *Siddiqia* Khan, Chawla & Saha, 1978 and *Longidoroides* Khan, Chawla & Saha, 1978 have either been synonymised with it and/or recognised as distinct in various review papers (Luc & Doucet, 1984; Coomans, 1985; 1996; Hunt, 1993; Siddiqi *et al*., 1993; Arias & Bravo, 1997; Escuer & Arias, 1997; Decraemer & Robbins, 2007). Recent molecular data based on ribosomal DNA (rDNA) sequences from 18S, ITS regions and the D2-D3 expansion segments of the 28S have been shown to be a useful diagnostic tool in the characterisation and phylogenetic relationships within Longidoridae, especially in cases where morphological characters may lead to ambiguous identification (De Luca *et al*., 2004, 2009; Neilson *et al*., 2004; Ye *et al*., 2004; He *et al*., 2005; Palomares *et al*., 2008; Gutiérrez-Gutiérrez *et al*., 2011; Pedram *et al*., 2012). Recent molecular phylogeny of dagger and needle nematodes based on the D2-D3 region of 28S and partial 18S genes has resolved three major clades: Clade I. *Longidorus* spp. and *Paralongidorus* spp.; Clade II. *Xiphinema americanum*-group including species of *Xiphidorus* (although not strongly supported in partial 18S gene); and Clade III comprising the remainder of the *Xiphinema* species (Gutiérrez-Gutiérrez *et al*., 2011). In this study, the tree topology analysis by Shimodaira-Hasegawa test of D2-D3 and partial 18S of a broad number of sequences did not refute the monophyly of

Xiphinema, which agreed with the results obtained by He *et al*. (2005). However, Gutiérrez-Gutiérrez *et al*. (2011), showed the paraphyly of *Paralongidorus* in a broad phylogeny, including all taxa with available sequence markers of Longidoridae, which also agreed with Rubtsova *et al*. (2001) and He *et al*. (2005) but disagreed with a more restricted study that included a smaller number of sequences conducted by Palomares-Rius *et al*. (2008). Nonetheless, in these studies the number of species and sequences of *Paralongidorus* considered were limited to the four available species (*P. litoralis*, *P. maximus*, *P. paramaximus*, *Paralongidorus* sp.). The Shimodaira-Hasegawa test (SH-test) is used to test the monophyly of taxa using a maximum likelihood test (Subbotin *et al*., 2005). The SH-test has advantages over other tests, such as the Kishino-Hasegawa and Templeton tests, because it compares simultaneously multiple topologies and corrects the corresponding *P* values to accommodate the multiplicity of testing (Buckley *et al*., 2001). Decraemer & Coomans (2007) concluded that, based upon: *i*) present information from available DNA sequences; *ii*) the wider range of variation of morphological features resulting from synonymisation of *Longidoroides* with *Paralongidorus*; and *iii*) the presence of transition forms between *Paralongidorus sensu* Siddiqi *et al.* (1993) and *Longidorus* with respect to the amphid structure (the main differential diagnostic feature for these genera), *Paralongidorus* should be synonymised with *Longidorus*. From the morphological point of view, a detailed study of the type material was carried out by Decraemer & Coomans (2007) on the structure of the amphid and found that several *Paralongidorus* species might actually belong to *Longidorus*. Nevertheless, the problem remains that most type material of *Paralongidorus* spp. (*e.g*., species from India) is no longer available. Similar remarks can be made concerning species of *Longidoroides*.

Longidorus and *Xiphinema* show a great biodiversity in Europe (Coomans *et al*., 2001). However, *Paralongidorus* has been detected more frequently in Asia and Africa, but only rarely in Europe, North and South America and Oceania (Coomans *et al*., 2001). In fact, Coomans (1985) considered a region located between India and South Africa before the separation of both continental plates as the probable centre of origin for *Longidorus* and *Paralongidorus*.

Four species of *Paralongidorus* (*viz*., *P. iberis* Escuer & Arias, 1997; *P. litoralis*; *P. maximus* and *P. paramaximus*) have been reported in the Iberian Peninsula in natural environments or in agricultural crops such as citrus (Macara, 1988; Escuer & Arias, 1997; Palomares-Rius *et al*., 2008). *Paralongidorus monegrensis* Escuer & Arias, 1997, described from northern Spain, was transferred to *Longidorus* by Decraemer & Coomans (2007) after

studying paratype material and demonstrating that the amphidial fovea, originally described as stirrup-shaped, appeared funnel-shaped in both male and female paratypes, and that the amphidial aperture, originally described as "distinct slit-like, 7-9 μm wide or about as long as half head width", appeared to be a pore.

During 2009-2010 extensive nematological surveys on Longidoridae in commercial vineyards and olive orchards, as well as several natural environments were done in southern Spain (Gutiérrez-Gutiérrez *et al*., 2010, 2011). All identified species belonged to *Longidorus* and *Xiphinema* and hitherto only one population of *Paralongidorus* was found in a sandy soil in the rhizosphere of stone pine in Bonares (Huelva Province). This population of *Paralongidorus* showed a very long body and stirrup-shaped amphidial fovea and morphologically resembled *P. epimikis* Dalmasso, 1969 and *P. litoralis* Palomares-Rius *et al*., 2008, a fact which led us to undertake a detailed morphological and molecular comparative study with previously reported data combined with molecular analyses to help clarify the phylogeny of the genus. These studies showed that the stone pine population differed from all known *Paralongidorus* species and is herein described as a new species.

The objectives of this work were: *i*) to characterise morphologically and molecularly the new species, *P. plesioepimikis* n. sp., from stone pine; *ii*) to study the phylogenetic relationships of this new species with *Paralongidorus* spp. and *Longidorus* spp. using sequences from the D2-D3 expansion regions of 28S rRNA and the partial 18S rRNA as inferred from Maximum Likelihood (ML) and Bayesian inference (BI) approaches; and *iii*) to test alternative topologies of the Longidoridae phylogenetic tree by constraining hypothetical monophyletic groups by using the SH-test.

Materials and methods

Nematode population

The nematode population used in this study was obtained from sandy soils at a depth of 10-50 cm from the rhizosphere of stone pine (*Pinus pinea* L.) at Bonares, Huelva Province, southern Spain. Nematodes were extracted by the sieving method described by Flegg (1967). Specimens for light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerin using Seinhorst's (1966) method. Specimens were examined using a Zeiss III compound microscope with differential interference contrast at powers up to ×1000 magnification. Measurements were done using a

camera lucida attached to a light microscope. The location of the pharyngeal gland nuclei follows Loof & Coomans (1972). For line figures, hand-made drawings were scanned and imported to CorelDraw software version 12 and redrawn.

For SEM studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold and observed with a JEOL JSM-5800 microscope (Abolafia *et al*., 2002).

DNA EXTRACTION, PCR AND SEQUENCING

For molecular analyses, two live nematodes were mounted on temporary slides in a drop of 1M NaCl containing glass beads. After taking measurements and photomicrographs, the slides were dismantled and DNA was extracted. Nematode DNA was extracted from single individuals and protocols for PCR were followed as described by Castillo *et al*. (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Castillo *et al*., 2003; He *et al*., 2005; Palomares-Rius *et al*., 2008). The ITS1 region was amplified using forward primer 18S (5'TTGATTACGTCCCTGCCCTTT-3') and reverse primer rDNA1 (5'-ACGAGCCGAGTGATCCACCG-3') as described in Wang *et al*. (2003). Finally, the 18S rDNA gene was amplified using the SSU_F_07 (5´-AAAGATTAAGCCATGCATG-3´), SSU_R_81 (5´- TGATCCWKCYGCAGGTTCAC-3´) and 13R (5'-GGGCATCACAGACCTGTTA-3') primers (http://www.nematodes.org/barcoding/sourhope/nemoprimers.html).

PCR products were purified after amplification using ExoSAP-IT (Affimetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and directly sequenced in both directions with the primers referred above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the SCAI, University of Córdoba sequencing facilities (Córdoba, Spain). Some sequences from other studied *Paralongidorus* spp. (*P. paramaximus* and *P. litoralis*) were included in order to increase the number of sequences for the topological test. The newly obtained sequences were submitted to the GenBank database under accession numbers JQ673403, JQ673405, JQ673407, JQ673409, and JQ673410 as indicated on the phylogenetic trees.

PHYLOGENETIC ANALYSIS

D2-D3 expansion segments of 28S and 18s-rRNA newly obtained sequences and sequences obtained from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were selected on the basis of their relationships in previously published large sub-unit (LSU) and short sub-unit (SSU) phylogenies (Holterman *et al*., 2006; Palomares-Rius *et al*., 2008; Coomans *et al*., 2012). The newly obtained and published sequences for each gene were aligned using ClustalW (Thompson *et al*., 1997) with default parameters. Sequence alignments were manually edited using BioEdit (Hall, 1999). Phylogenetic analysis of the sequence data sets were performed with ML using PAUP * 4b10 (Swofford, 2003) and BI using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained using the program JModelTest ver. 0.1.1 (Posada, 2008) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites and the gamma distribution shape parameters and substitution rates in the AIC were used in phylogenetic analyses. BI analysis under GTR + G + I model for both genes was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView program (Page, 1996). In ML analysis, the estimation of the support for each node was made using a bootstrap analysis with 100 fast-step replicates. In order to test alternative tree topologies by constraining hypothetical monophyletic groups, we performed SH-test as implemented in PAUP (Swofford, 2003) using RELL option. In total, 44 and 43 species were selected from *Xiphinema*, *Xiphidorus*, *Longidorus* and *Paralongidorus*, using *Tylencholaimus mirabilis* as outgroup, for the D2-D3 expansion segments of 28 rRNA and partial 18S, respectively. Species were selected from guidelines in the clades formed in the broader phylogeny of Longidoridae performed by Gutierrez-Gutierrez *et al.* (2011). The tested hypothetical monophyletic groups were performed using ML and included *Xiphidorus*, *Xiphinema* and *Paralongidorus*.

Results

***Paralongidorus plesioepimikis*[*] n. sp.**

(Figs 1-4)

MEASUREMENTS

See Table 1.

Female

Body very long and rather robust, slightly tapering towards posterior end and also slightly anteriorly, usually assuming an open C-shaped when heat relaxed. Cuticle smooth under LM, 5.5 (4.5-6.0) µm thick, 12.9 (10.5-18.0) µm thick at tail end, and marked by very fine superficial transverse striae mainly in tail region, as shown by SEM. Lip region widely rounded in lateral view, clearly set off by a clear constriction followed by a depression posterior to amphidial aperture, 13.7 ± 0.7 (12.5-14.5) µm high. SEM observations showing protruding inner labial papillae and outer labial papillae surrounded by a cuticular ring. Amphidial fovea very large, stirrup-shaped, with conspicuous slit-like aperture *ca* three-fourths as wide as lip region. Cephalic papillae appearing as small apertures, each located just anterior to a distinct cephalic lobe 2.5-3.0 µm long. Stylet guiding ring single, 7-8 µm wide, located 1.3 ± 0.1 (1.2-1.4) lip region diam. from anterior end. Lateral chord 18 (14-23) µm wide at mid-body or 22% of corresponding body diam. Odontostyle very long and narrow, 2.8 (2.4-3.3) times as long as odontophore, straight or slightly arcuate, *ca* 3.0-3.5 µm wide towards its base, odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling narrower part of pharynx slightly posterior to odontophore base, 4.9 (3.8-6.1) body diam. at neck base from anterior end. Anterior slender part of pharynx usually with looped region overlapping basal bulb. Basal bulb cylindrical, 162 ± 14.4 (143-186) µm long or *ca* one-third to one-fifth of neck length, 37.5 (31-48) µm diam. Dorsal pharyngeal gland nucleus in anterior part of bulb, 12.5-23.0 µm posterior to gland outlet, one ventro-sublateral pair of nuclei near middle of bulb, larger than dorsal nucleus. Glandularium 146.0 ± 16.7 (128-167) µm long. Cardia conoid-rounded, 17.6 ± 4.5 (14-25) µm long. Reproductive system with both genital branches equally developed, each branch 382-494 µm long, with reflexed ovaries of very variable length, vulva in form of a transverse slit, located slightly anterior to mid-body, vagina perpendicular to body axis, 38-44 µm long, or 45-51% of corresponding

[*] The species epithet refers to a compound name from Greek word: *plesios* = near, and *epimikis* the closet species of the genus *Paralongidorus*.

body diam., surrounded by well developed muscles. Uteri 123-161 µm long, without sperm cells in all females examined; well developed sphincter between uterus and oviduct. Anterior and posterior oviduct of similar size. Anterior ovarium 212.0 ± 83.2 (139-341) µm long, posterior ovarium 226.0 ± 70.1 (163-337) µm long, both with a single row of oocytes. Gravid females with only one egg in one gonoduct, 262-344 µm long × 71-72 µm diam. Prerectum very variable in length, 4.0-8.1 anal body diam. long and rectum 0.6-0.7 anal body diam. long, anus a small rounded slit. Tail short, barely dorsally convex-conoid, with rounded terminus, bearing three pairs of caudal pores.

Male

Not found.

Juveniles

All four juvenile stages were found and distinguished by relative lengths of body and functional and replacement odontostyle (Table 1), (Robbins *et al*., 1995, 1996). Resembling adults in most respects except for size and development of reproductive system. First-stage juveniles (J1) characterised by a conoid-rounded tail, with a digitate rounded mucro, 8.0 ± 0.5 (7.5-8.5) µm long (Fig. 2 H), odontostyle length *ca* 120 µm long, and shorter distance from anterior end to stylet guiding ring than that in adult stages. However, morphology in all three juvenile stages (except for undeveloped genital structures) similar to that of female, including bluntly rounded tail shape of third- and fourth-stage juveniles which was, yet differed in shorter distance from anterior end to guiding ring.

TYPE HABITAT AND LOCALITY

Rhizosphere of stone pine (*Pinus pinea* L.) from Bonares, Huelva Province, southern Spain (37º17’37.92’’ N latitude, 6º39’42.17’’ W longitude).

TYPE MATERIAL

Holotype female (slide H148-12) and 14 female paratypes (slides H148-01- H148-20) deposited in the Nematode Collection of the Institute for Sustainable Agriculture, IAS-CSIC,

Córdoba, Spain. Two female and one J1 paratypes deposited at each of the following nematode collections: Royal Belgian Institute of Natural Sciences, Brussels, Belgium; Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (CNR), Sezione di Bari, Bari, Italy; USDA Nematode Collection, Beltsville, MD, USA. Specific D2-D3, partial 18S, and ITS1-rRNA sequences deposited in GenBank with accession numbers JQ673403, JQ673405, and JQ673407, respectively.

DIAGNOSIS AND RELATIONSHIPS

Paralongidorus plesioepimikis n. sp. is characterised by a very long body (9.71-14.11 mm), a lip region widely rounded with a clear constriction followed by a depression and bearing a very large stirrup-shaped, amphidial fovea, with conspicuous slit-like aperture, a very long and flexible odontostyle *ca* 215 µm long, stylet guiding ring located at *ca* 40 µm from anterior end, vulva rather anterior (33-38%), tail short, dorsally convex-conoid, with rounded terminus, bearing three pairs of caudal pores, male absent, and specific D2-D3, ITS1, and partial 18S-rRNA sequences. According to the polytomous key of Escuer & Arias (1997), the new species has the following matrix code: A1, B1, C3, D2, E1, F6, G7, H2, I2, J12, K6, L34, M3, N-, O-.

On the basis of amphidial fovea, lip region, body and odontostyle length, ratios a, c, and c', distance from oral aperture to guiding ring, bulb length, tail length and shape, lack of males and tail morphology of the J1, *P. plesioepimikis* n. sp. is close to *P. australis* Stirling & McCulloch, 1984, *P. epimikis*, *P. deborae* (Jacobs & Heyns, 1982) Luc & Doucet, 1984, *P. iranicus*, *P. litoralis*, *P. maximus* and *P. rex* Andrássy, 1986. Morphologically and morphometrically, *P. plesioepimikis* n. sp. can be distinguished from these species by several features as shown in Table 2. From *P. epimikis* it differs by a longer oral aperture to guiding ring distance, lip region diam., shape of amphidial fovea and basal bulb length (Table 2). Also, although the J1 tail shape of the new species and *P. epimikis* are rather similar, both species differ by ratio c' which is 1.4 (1.3-1.6) *vs* 2.22 (2.22-2.23) and tail length of 39.8 (38-45) *vs* 50 (49-50) µm. Similarly, second- and fourth-stage juveniles of the new species and *P. epimikis* are similar except for a slightly different ratio a and the oral aperture to guiding ring distance (Table 2). The application of the polytomous principle for the identification of *Paralongidorus* species is a useful tool, although because of the great number of species it can be difficult to separate species as there is overlapping of various characters states. In fact, no

single morphometric character could unambiguously separate the species as demonstrated for the species group in Table 2.

MOLECULAR CHARACTERISATION OF *PARALONGIDORUS PLESIOEPIMIKIS* N. SP. AND PHYLOGENETIC POSITION WITHIN *LONGIDORUS* AND *PARALONGIDORUS*

Amplification of the partial 18S, D2-D3 expansion segment of 28S rDNA and ITS rRNA from *P. plesioepimikis* n. sp. yielded a single fragment of *ca* 1700, 800, and 1100 bp, respectively. Sequence variability for the D2-D3 region among the *Paralongidorus* sequences retrieved from GenBank and *P. plesioepimikis* n. sp. varied from 21 to 123 nucleotides (2.8-16.4%). The 18S rRNA gene showed a lower diversity than D2-D3 segments of 28S rRNA, varying from 1 to 21 nucleotides (0.06-1.3%) for *P. plesioepimikis* n. sp. Sequences from ITS1 rDNA among *Paralongidorus* species showed the highest diversity in comparison to the other rDNA markers, varying from 162 to 417 nucleotides (16.4-45.4%) for *P. plesioepimikis* n. sp. The scarce homology with other *Paralongidorus* species and the few sequences deposited in GenBank did not allow the phylogenetic analysis of ITS1 rDNA. Using these three molecular markers, the new species could be clearly separated from all other sequenced *Paralongidorus* spp., with a lower number of differences with the partial 18S gene in comparison to the other molecular markers.

Phylogenetic trees reconstructed by the BI method for the two rRNA genes (D2-D3 expansion regions of 28S rRNA gene and the partial 18S rRNA) are presented in Figures 5 and 6, respectively. The phylogenetic trees obtained were generally congruent with those given by Pedram *et al*. (2012) for D2-D3 of 28S and 18S genes, respectively, with the exception of the position of some poorly supported clades (Figs 5, 6). No significant difference in topology was obtained using the ML or BI approach for both markers and only a few species in some minor clades with low bootstrap values were not congruent with the general topology tree. *Paralongidorus plesioepimikis* n. sp. formed a well supported clade with the rest of *Paralongidorus* spp. with the exception of *P. bikanerensis* (JN032584) in BI and ML trees generated from the D2-D3 of 28S sequence dataset (Fig. 5); the closest related species were *P. litoralis* (EU026155) and *P. paramaximus* (EU026156) (Fig. 5). However, the position of *P. bikanerensis* is poorly supported (Fig. 5), while the rest of the *Paralongidorus* sequences clustered as an additional clade of the genus *Longidorus*. Similarly, *P. plesioepimikis* n. sp. is grouped together with *P. paramaximus* (EU026157) and *P. litoralis* (EU026159) in BI and Ml trees generated from the partial 18S (Fig. 6). All *Paralongidorus*

spp. clustered together except for *P. bikanerensis* (JN032586) (Fig. 6). Trees generated using D2-D3 and partial 18S using BI and ML (Figs 5, 6) showed a congruent position of *P. plesioepimikis* n. sp. The clade including *P. plesioepimikis* n. sp. grouped species characterised by a long body and odontostyle, lip region set off by a clear constriction followed by a depression, and a very large stirrup-shaped amphidial fovea with conspicuous slit-like aperture.

The tree topologies studied, using selected taxa from Longidoridae and one outgroup (*T. mirabilis*), by SH-test did not refute the monophyly of *Xiphinema* even though it was split into two major clades (Gutiérrez-Gutiérrez *et al*., 2011) (D2-D3 region, $P = 0.077$; and partial 18S, $P = 0.471$) (Table 3). *Paralongidorus* was accepted as a group outside *Longidorus*, using the D2-D3 region and the partial 18S gene (D2-D3 region, $P = 0.061$; partial 18S, $P = 0.417$) (Table 3). Finally, *Xiphidorus* showed an accepted position outside of *Xiphinema* (D2-D3 region, $P = 0.343$; partial 18S, $P = 0.471$) (Table 3). However, the influence of the only sequence from *P. bikanerensis* using the limited number of *Paralongidorus* species available rejected the monophyly of *Paralongidorus* in both options studied (including it outside or inside *Longidorus*). These results partially disagree with those obtained by Gutierrez-Gutierrez *et al*. (2011), except for the monophyly test of *Paralongidorus* using the partial 18S gene. However, our results agree with those obtained by He *et al*. (2005) and Palomares-Rius *et al*. (2008). These differences could be related to the different number of sequences used in this study and the tree calculation for comparison of the different hypothesis tested by the SH-test (ML in the case of this study). Maximum Likelihood tree construction using the nucleotide substitution model are more accurate in phylogenetic reconstruction than MP (Gadagkar & Kumar, 2005) and consequently fewer differences between trees could be detected. Interestingly, a former *Longidoroides* species (*P. bikanerensis*) showed a remarkable position outside of *Paralongidorus* in all phylogenetic analyses. However, a careful examination of the amphids by Decraemer & Coomans (2007), and SEM studies by Pedram *et al*. (2012) considered this species to be a member of *Paralongidorus*. The possibility of studying molecularly more specimens for *Longidoroides* or former member of this group would be of interest in order to clarify this situation.

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Table 1. Morphometrics of females and juveniles of *Paralongidorus plesioepimikis* n. sp. from southern Spain. All measurements are in µm (except for L) and in the form: mean ± s.d. (range)*.

Character	Female		J1	J2	J3	J4
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	Paratypes
n	–	20	6	9	5	3
L (mm)	12.43	11.68 ± 1068 (9.71-14.11)	2.03 ± 0.144 (1.79-2.21)	2.64 ± 0.3 (2.43-2.99)	4.57 ± 0.28 (4.30-4.89)	8.62 ± 1.33 (7.09-9.48)
a	168.0	141.6 ± 11.7 (121.3-168.0)	50.4 ± 3.6 (45.8-53.9)	62.7 ± 1.4 (61.3-64.3)	80.3 ± 5.6 (75.4-87.9)	111.0 ± 8.4 (101.3-116.2)
b	16.9	17.0 ± 1.6 (13.6-19.1)	6.4 ± 1.0 (5.2-7.9)	6.3 ± 0.8 (5.9-7.6)	8.3 ± 0.5 (7.7-8.9)	12.9 ± 1.8 (11.5-14.9)
c	336.0	332.7 ± 38.9 (269.6-403.3)	51.0 ± 3.7 (45.8-52.4)	80.9 ± 6.4 (73.7-88.9)	120.9 ± 8.5 (108.0-127.6)	239.6 ± 37.2 (197.0-265.6)
c´	0.8	0.7 ± 0.05 (0.7-0.8)	1.4 ± 0.1 (1.3-1.6)	1.0 ± 0.1 (0.9-1.1)	0.8 ± 0.06 (0.8-0.9)	0.6 ± 0.03 (0.6-0.7)
V or T	35	35.8 ± 1.1 (33-38)	–	–	–	–
G_1	3.6	4.3 ± 1.4 (3.2-8.0)	–	–	–	–
G_2	3.6	4.3 ± 1.4 (3.2-8.0)	–	–	–	–
Odontostyle	206	215 ± 7.3 (202-227)	118 ± 4.1 (113-120)	137 ± 5.0 (132-143)	164 ± 6.7 (154-173)	185 ± 8.7 (175-191)
Replacement odontostyle	–	–	134 ± 5.3 (126-139)	161 ± 5.3 (156.0-169.0)	182 ± 5.7 (177-190)	210 ± 10.6 (198-218)
Odontophore	84.0	76.4 ± 5.2 (66.0-87.0)	55.4 ± 3.0 (52.0-60.0)	67.6 ± 4.4 (65.0-74.0)	61.2 ± 2.1 (58-64)	68.3 ± 3.1 (65-71)
Lip region diam.	32.0	31.7 ± 1.4 (29.0-34.0)	15.5 ± 0.5 (15.0-16.0)	19.5 ± 1.9 (17.0-21.0)	25.0 ± 0.6 (23.0-26.0)	29.5 ± 0.7 (29.0-30.0)
Oral aperture-guiding ring	41.0	40.3 ± 1.9 (36.5-44.0)	23.0 ± 1.3 (21.0-24.0)	25.9 ± 2.2 (23.0-27.0)	31.7 ± 0.6 (31.0-32.5)	37.3 ± 4.0 (33.0-41.0)
DO	12.3	11.4 ± 3.0 (8.3-14.2)	–	–	–	–
DN	25.2	22.8 ± 3.6 (16.0-26.8)	–	–	–	–
SN_1 & SN_2	55.4	55.7 ± 1.1 (54.2-57.1)	–	–	–	–
SO_1 & SO_2	85.7	85.2 ± 1.1 (83.4-86.2)	–	–	–	–
Pharynx length	734	691 ± 79.0 (593-869)	323 ± 44.1 (252-368)	421 ± 28.3 (394-461)	554 ± 49.1 (481-594)	667 ± 72.4 (617-750)
Tail length	37.0	35.1 ± 2.1 (32.0-39.0)	39.8 ± 2.8 (38.0-45.0)	32.8 ± 4.1 (28.0-38.0)	37.9 ± 2.5 (35.0-40.0)	36.0 ± 1.0 (35.0-37.0)

*Abbreviations as defined in Jairajpuri & Ahmad (1992).

Table 2. Differential morphometrics of *Paralongidorus plesioepimikis* n. sp. All measurements are in µm, except L in mm.

Character	*plesioepimikis* n. sp.	*australis*	*deborae*	*epimikis*	*iranicus*	*litoralis*	*maximus*	*rex*
L	9.7-14.1	7.6-10.6	7.5-11.5	9.9-10.5	7.8-11.4	7.5-10.1	7.6-12.4	9.6-9.8
a	121-168	85-116	156-243	184-211	101-138	114-164	72-133	106-111
c	270-403	255-426	243-327	309-350	221-315	235-335	178-320	230-250
c’	0.6-0.8	0.48-0.59	0.8-1.0	0.82-0.84	0.5-0.7	0.64-0.83	0.4-0.6	0.5-0.6
Odontostyle length	202-227	146-170	154-168	205-216	153-184	169-206	152-187	178-180
Oral aperture-guiding ring	37-44	58-70	34-38	24-31	32-39	32-37	37-47	38
Lip region diam.	29-34	18-21	25-27	44-46	25-30	25-30	34-39	32-33
Amphidial fovea shape	Stirrup	Funnel	Stirrup	Funnel	Stirrup	Stirrup	Stirrup	Funnel
Basal bulb length	143-186	114-146	123-154	108-114	120-150	112-143	–	–
Tail length	32-39	21-35	29-38	29-34	25-37	27-34	36-41	40-45
Tail shape	dorsally convex-conoid	broadly rounded	convex-conoid	conoid-rounded	broadly rounded	bluntly rounded	bluntly rounded	conoid-rounded
Male	not found	present	present	present	present	present	rare	not found
c’ in J1	1.3-1.6	0.48-0.60	–	2.22-2.23	–	1.4-1.8	1.06-1.25	1.1-1.3
Tail length in J1	38-45	14-15	–	49-50	–	41-46	37-44	29-36

Table 3. Results of the SH-tests for alternative hypotheses using ML trees.

Topologies and hypothesis tested	D2-D3			18S		
	-*Ln*L	Difference of -*Ln*L	*P* value	-*Ln*L	Difference of -*Ln*L	*P* value
ML tree	10972.12	best	-	5760.16	0.0000	0.899
All *Xiphinema* species constrained into one group	11016.93	44.81	0.077	5761.34	1.19	0.471
Paralongidorus constrained to be a group outside *Longidorus*	11008.96	36.83	0.061	5761.17	1.02	0.417
Paralongidorus constrained to be a group outside *Longidorus*; excluding *P. bikanerensis* and joining it to *Longidorus*	11146.31	174.18	0.000*	5760.16	best	-
Paralongidorus constrained to be a group outside *Longidorus*; excluding *P. bikanerensis* and excluding it from *Longidorus*	11202.96	230.83	0.000*	5761.17	1.02	0.417
Xiphidorus constrained to be a group outside of all *Xiphinema* species	10990.97	18.84	0.343	5761.34	1.19	0.471

*$P < 0.05$ indicates the significant differences between the two inferred tree topology.

Figure legends

Fig. 1. Paralongidorus plesioepimikis *n. sp. A: Neck region; B: Lip region; C: Detail of basal pharyngeal bulb; D: Vulval region; E: Detail of genital branches; F, G: Female tail.*

Fig. 2. *Light micrographs of* Paralongidorus plesioepimikis *n. sp. A: Female anterior region; B-D: Lip regions showing amphidial fovea at different focus; E: Detail of basal bulb; F: Detail of anterior and posterior genital branches; G: First-stage juvenile anterior region; H-K: Tail of J1, J2, J3, and J4, respectively; L: Female tail. Abbreviations: a = anus; af = amphidial fovea; gr = guiding ring; ost = odontostyle; ov = ovary; rost = replacement odontostyle; V = vulva. (Scale bars A, I, O, P = 50 µm; B, C, F, G, H, J-N= 25 µm; D-E= 10 µm.)*

Fig. 3. SEM *micrographs of* Paralongidorus plesioepimikis *n. sp. A-D: Female anterior end in lateral and ventro-lateral view showing cephalic lobe (cl), and amphidial aperture (af); E, F: Female tail, lateral view showing caudal pores; G: Detail of anus. (Scale bars: A, E-G = 50 µm; B-D = 20 µm.)*

Fig. 4. *Relationship of body length with length of functional and replacement odontostyle (ost and rost, respectively) length in all detected developmental stages to mature females of* Paralongidorus plesioepimikis *n. sp.*

Fig. 5. *Phylogenetic relationships of* Paralongidorus plesioepimikis *n. sp. within* Longidorus *and* Paralongidorus *for D2 and D3 expansion segments of 28S rRNA. Bayesian 50% majority rule consensus trees as inferred from D2 and D3 expansion segments of 28S rRNA sequences alignments under the* GTR + G + I *model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences in this study are in bold letters.*

Fig. 6. *Phylogenetic relationships of* Paralongidorus plesioepimikis *n. sp. within* Longidorus *and* Paralongidorus *for 18S rRNA. Bayesian 50% majority rule consensus trees as inferred from partial 18S rRNA gene sequences alignments under the* GTR + G + I *model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences in this study are in bold letters.*